1. PURPOSE

This procedure provides instructions for delivering IVT-synthesized RNA products into eukaryotic cells by cationic liposome-mediated transfection.

2. SCOPE

This SOP covers the sample preparation, method execution, and basic result interpretation for the delivery of RNA, synthesized by IVT, into eukaryotic cells using the cationic liposome transfection agent Lipofectamine RNAiMAX, as performed by the RNAcore at the Houston Methodist Research Institute.

3. AUDIENCE/RESPONSIBILITIES

All personnel working on RNA quality control assessment and associated materials as part of the RNAcore's research operations within the Houston Methodist Research Institute must abide by this SOP.

4. ABBREVIATIONS/DEFINITIONS

4.1 Abbreviations

°C	Celsius (unit of temperature)
CO_2	Carbon Dioxide
IVT	*in-vitro* Transcription
LFA	Lipofectamine
LN_2	Liquid Nitrogen
µg	Microgram (unit of mass)
µl	Microliter (unit of volume)
mL	Milliliter (unit of volume)
MM	Master Mix
mRNA	Messenger RNA
nGFP	Green Fluorescent Protein
RNA	Ribonucleic Acid
RT	Room Temperature

4.2 Definitions

Lipofectamine Negative Control: A reaction tube that contains Lipofectamine but no RNA. This control is necessary to ensure that the effect observed on the cells is due to the IVT RNA and not the Lipofectamine. If the Lipofectamine control causes a decrease in cell viability, the concentration of Lipofectamine in the treatment should be optimized.

Master Mix*:* A single mixture of the reagents that after homogenization is aliquoted into multiple reaction tubes. This ensures equal concentration of reagents across all reaction tubes, reducing variability.

Positive Control: A reaction tube that contains LFA and a reporter IVT mRNA whose expression by the cell is readily observable (*e.g.*, nGFP).

Triplicate*:* To perform a reaction in three's to be able to generate data that allow for statistical analysis

Room temperature*:* For the purpose of this process, it is 20–25°C.

Untreated Negative Control*:* A reaction tube that contains no RNA or Lipofectamine, which should yield no effect on the cells. This control is necessary to ensure that the effect observed on cells is due to the treatment.

5. MATERIALS AND EQUIPMENT

5.1 Materials

Description	Source [Cat. No.]	Storage Conditions
RNA Product(s)	Provided by RNAcore	- 80°C Ultralow Freezer
Eukaryotic Cells to be treated	Depends on study	LN_2
Lipofectamine® RNAiMAX transfection Reagent	Invitrogen (13778150)	Refrigerated (4°C)
Opti-MEM® I Reduced Serum Medium	Gibco (31985062)	Refrigerated (4°C)
Culture Media with serum and antibiotics	Any in use	Refrigerated (4°C)
6-well plate	Any in use	RT
1.5mL Microfuge Tubes, RNase-free	Any in use	RT

Description	Source [Cat. No.]	Storage Conditions
1.5mL Tube Racks	Any in use	RT
Micropipette Tips, nuclease-free (various sizes)	Any in use	RT
Sharps Container	Any in use	RT
Ice bucket	Any in use	RT
Clean Ice	Any in use	From Icemaker Freezer
Disinfectant Agent (*e.g.*, 70% isopropanol)	Any in use	RT

5.2 Equipment

Description	Model	Location
Biosafety Cabinet or Laminar Flow Hood	Any in use	Cell Culture Room
CO_2 Incubator (set at 5% CO_2, 37°C)	Any in use	Cell Culture Room
Micropipettors (various sizes)	Any in use	Cell Culture Hood
Vortex Mixer	Any in use	Cell Culture Hood

6. SAFETY

6.1 Electrical Hazards

6.1.1 Never operate the instrument from a power outlet without a ground connection. It could result in injury from electric shock.

6.1.2 Do not operate the instrument in the presence of flammable gases or fumes. Operation of any electrical instrument in such an environment is hazardous.

6.2 Chemical & Biological Safety

6.2.1 Wear appropriate personal protective equipment and observe safe handling practices when working with reagents and samples.

- The reagents used in this procedure contain components that are irritant and toxic. Refer to material safety data sheets.

6.2.2 Dispose of hazardous waste (*e.g.*, tubes, waste solutions) into appropriate waste containers.

6.2.3 Clean up and decontaminate any surfaces contaminated by spills or splashes.

6.3 Mechanical Safety

6.3.1 Observe appropriate safety practices when using equipment with high speed moving parts, such as centrifuges and vortexes.

- Do not interfere with moving rotors; do not stop by hand.
- Avoid the dispersion of aerosols.

6.3.2 Observe appropriate precautions when handling and discarding sharps (micropipette tips are considered sharps at The Methodist Research Institute). Use adequate sharps disposal containers.

7. PROCEDURE

7.1 Procedural Notes

NOTE A: Perform all manipulations in the biosafety cabinet or laminar flow hood with proper techniques and proper personal protective equipment.

NOTE B: Ensure that the work surfaces have been cleaned and disinfected prior to beginning work.

NOTE C: All tools such as pipettors must also be wiped with disinfectant agent prior to introduction into biosafety cabinet or laminar flow hood.

NOTE D: Discard waste from cell-handling operations into an appropriate biohazardous waste container.

NOTE E: Refer to ***Appendix A*** for a summary diagram of the transfection procedure.

7.2 Pre-requisites

7.2.1 Forty-eight hours before transfection, seed cells in a 6-well plate such that they are 75-80% confluent by the time of transfection.

7.2.2 Incubate cells in 2mL of media with serum and antibiotics, appropriate to the type of cells being transfected, for 48 hours at 5% CO_2, 37°C.

7.3 Setup for Transfection of Cells in a 6-Well Plate

7.3.1 Equilibrate Opti-MEM Media to room temperature for about thirty minutes prior transfection.

7.3.2 Place IVT RNA and Lipofectamine (LFA) in a bucket of ice right before the transfection.

7.3.3 Label fresh 1.5mL tubes for each set of conditions. Triplicates are recommended.

a. Tubes should be labeled for the following conditions: Untreated Negative control (Un-), Lipofectamine Negative Control (LFA-), and each of the IVT RNA transfection samples. nGFP mRNA may be used as positive control.

b. In this SOP, triplicates for untreated negative control, LFA negative control, and IVT RNA are depicted.

7.3.4 Prepare the Transfection Master Mix, as detailed:

a. Label a fresh 1.5mL tube "MM" for Master Mix.

b. Add Opti-MEM Media to the MM reaction tube. To determine the amount of Opti-MEM Media to add, take the total number of wells and multiply by 100µl.

c. Take the Lipofectamine from the bucket of ice and add LFA to the MM reaction tube. To determine the amount of LFA to add, take the total number of wells and multiply by 3µL.

d. Once LFA is added to the MM reaction tube, place the LFA back on ice.

e. Mix the MM reaction tube by vortexing and hold at room temperature.

7.3.5 Prepare the Untreated Negative Control tubes by adding only Opti-MEM Media.

a. To determine the amount of Opti-MEM Media to add, take the number of wells for this condition and multiply by 200µl.

7.3.6 Prepare the LFA Negative Control Transfection tubes by adding only Opti-MEM Media.

a. To determine the amount of Opti-MEM Media to add, take the number of wells for this condition and multiply by 100µl.

7.3.7 Prepare the IVT RNA Transfection tubes, as follows:

a. Add 100µL of Opti-MEM Media to each of the pre-labeled IVT RNA transfection tubes.

- To determine the total amount of Opti-MEM Media to add, multiply the number of wells for this particular condition treatment by 100µL.

b. Take the IVT RNA from the bucket of ice and add 1-2 µg into the Opti-MEM Media aliquoted into the pre-labeled IVT RNA Transfection tubes in the previous step.

- To determine the total amount of IVT RNA to add, multiply the number of wells for this condition by the desired concentration of IVT RNA that is needed per well. IVT RNA concentration needed per well varies depending on cell type and the type of IVT RNA; 1-2 µg per well is recommended.

c. Place IVT RNA back on ice.

d. Note: DO NOT vortex the IVT RNA Transfection tubes.

7.3.8 Add the Master Mix to all Transfection tubes, EXCEPT the Untreated Negative Control.

a. Add Master Mix to the diluted IVT-RNA Transfection labeled tubes in a 1:1 ratio. To determine how much Master Mix to add in the IVT-RNA Transfection labeled tubes, multiply the number of wells for this condition by 100µl.

b. Add Master Mix to the diluted LFA Negative Control Transfection labeled tube in a 1:1 ratio. To determine how much Master Mix to add to the "LFA-" labeled tube, multiply the number of wells for this condition by 100µl.

c. Mix by vortexing.

7.3.9 Incubate all Transfection tubes, including controls, for 10 minutes at room temperature.

7.3.10 Prepare cells for the transfection.

a. Remove the 6-well plate from the CO_2 incubator. Label the wells for triplicates of each corresponding condition.

b. Remove 1mL of media from each well so that the final volume per well after addition of reagents is 1mL.

7.4 Transfection of Cells in a 6-Well Plate

7.4.1 Add the transfection reagent mixtures from *Step 7.3.8* to cells.

a. After the 10 minute RT incubation, add 200µL of each transfection reaction tube mixture drop-wise to each corresponding well with cells.

7.4.2 Place plate(s) back into the CO_2 incubator (which is set to 37°C, 5% CO_2).

7.4.3 Place Lipofectamine back into the 4°C Refrigerator and the IVT RNA in the -80°C ultrafreezer.

7.4.4 Four hours after transfection, remove the plate(s) from the CO_2 incubator, remove the transfection medium, and replace with 2mL/well of fresh growth medium appropriate for culture of the cell type being treated (this medium may include serum, antibiotics, or cytokines). Place plate(s) back into the CO_2 incubator.

7.5 Verification of Transfection

7.5.1 RNA should be internalized approximately 1-2 hours post-transfection and RNA should be expressed approximately 4-6 hours post-transfection.

7.5.2 Inspect the negative control cell wells to ensure there were no interferences in the process. If there are signs of gross cell death or morphological changes not typical of your cell type, the transfection should be questioned.

7.5.3 If using a positive control, such as nGFP mRNA, signs of RNA expression should be grossly visible via fluorescence microscopy after 6 hours, post-transfection.

7.5.4 After verification of successful transfection, the transfected cells can be further analyzed or assayed as desired.

8. APPENDICES

Appendix A: Summary of transfection procedure for a single well (6-well plate)

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Seed and culture cells (48 hours, 5% CO_2, 37°C),
such that they are 75-80% confluent at the time of transfection.

↓

Prepare then combine Transfection Reagent
Solutions (Master Mix and IVT RNA)

Transfection Master Mix	
Component	*Volume per Well*
LFA	3 uL
OptiMEM	100 uL

+

RNA Transfection Solution	
Component	*Volume per Well*
IVT RNA	1 - 2 ug
OptiMEM	100 uL

↓ 10 minutes, RT

Add 200 µL of transfection reagent mixture per well, drop-wise

↓ 4 hours, 37°C, 5% CO_2

Replace transfection medium with growth medium
appropriate for culture of the particular cell type.

9. AUTHORITATIVE REFERENCES

9.1 Lipofectamine® RNAiMAX Transfection Reaction Protocol from Life Technologies

(available at https://www.thermofisher.com/order/catalog/product/13778150)

10. APPROVAL

J Cantafeda — 28Jul16

RNAcore Director or Designee — Date

11. VERSION HISTORY

Version	Effective Date	Summary of Changes
01	28Jul16	Created document.